Glucosamine loaded injectable silk-in-silk integrated system modulate mechanical properties in bovine ex-vivo degenerated intervertebral disc model

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A B S T R A C T

Injectable hydrogels offer a tremendous potential for treatment of degenerated intervertebral disc due to their ability to withstand complex loading, conforming precisely to the defect spaces and eliminating the need for invasive surgical procedures. We have developed an injectable hydrogel platform of N-acetylglucosamine (GlcNAc) loaded silk hollow spheres embedded in silk hydrogel for in situ therapeutic release and enhanced mechanical strength. The assembled silk hydrogel provided adequate structural support to the ex vivo degenerated disc model in a cyclic compression test at par with the native tissue. Spatiotemporal release of GlcNAc in a controlled manner from the silk hollow microspheres trigger enhanced proteoglycan production from ADSCs embedded in the composite system. Role of MAPK and SMAD pathways in increasing proteoglycan production have been explored by immunohistological analysis as a result of the action of GlcNAc on the cells, elucidating the potential of injectable silk microsphere-in-silk hydrogel for the regeneration of degenerated disc tissue.

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1. Introduction

Nucleus pulposus (NP) of intervertebral disc (IVD) is a highly hydrated (70–90%) tissue that contains mainly proteoglycans and collagen type II [1]. The IVD tissue provides support and dissipates the axial compressive forces in spinal joints. With aging process, disc degeneration pathophysiology initiates in the NP due to loss of cellularity, decreased water content and loss of proteoglycans from the extracellular matrix [2]. These incidents alter the swelling properties of the NP that translates into the reduction in disc height and poor ability to absorb the mechanical pressure applied on the spine [3]. To date, there is no successful treatment strategy available for degenerative disc diseases.

Hydrogels have been implanted into the degenerated discs in a minimally invasive manner; in an effort to reduce the surgery time and post-surgical trauma. Fibrin [4–6], hyaluronan [7,8], collagen [9,10] and chitosan [11–13] based hydrogels have been used as carriers of cells and for the delivery of biochemical cues for promoting the regeneration of NP tissue. Various growth factors, such as TGF-β1 [14] and BMP-2 [14,15], delivered along with cells, have been shown to enhance ECM deposition. The presence of these factors has resulted in increased deposition of ECM components; however, a major disadvantage is that it has also lead to the ossification of the adjacent AF region [16]. Gellan gum hydrogels have been found to maintain native phenotype of NP cells [17,18], but the mechanical properties of the crosslinked hydrogels differed from the native NP tissue. In both the aforementioned conditions, the hydrogel implant may fail to maintain biomechanical stability of the disc and hence promote further disc degeneration. Most of these hydrogels developed for supporting cell growth and growth factor delivery inside the degenerated tissue have not been successful in restoring the viscoelastic and mechanical properties of the NP tissue. This challenge can be addressed by designing a
hydrogel system that can encapsulate cells and deliver biochemical cues; while being robust enough to mimic the viscoelastic properties of the native tissue.

Silk was the material of choice because of its known cellular compatibility and low immunogenicity [19,20]. Silk fibron hydrogels have been used as injectable materials for healing confined critical size cancellous defects due to their excellent intrinsic mechanical behavior [21]. Injectable sonication-induced silk hydrogels have been used to deliver VEGF and BMP-2 for the elevation of the maxillary sinus floor [22]. Silk microspheres and nanospheres have been fabricated from silk/PVA blend films, by dissolution of silk-PVA films in water and their subsequent ultrasonication [23]. This process generated spheres of non-uniform sizes [23]. Some studies have demonstrated the use of silk hydrogels in combination with other synthetic polymers for NP tissue engineering. A silk fibron-fibrin-hyaluronic acid composite gel was used for NP tissue engineering [24], where 1% and 2% silk hydrogels were used in conjugation with fibrin and hyaluronic acid for controlling the degradation rate of the scaffolds. The silk fibron helped in enhancing the mechanical properties of the fibrin/hyaluronic acid hydrogels, which still lacked the viscoelastic properties and injectability that are a pre-requisite in NP tissue regeneration. Another study demonstrated that the injectable silk fibron/polyurethane composite hydrogels augment NP tissue [25]. In context of the current literature, our study demonstrates that the use of synthetic polymers, like polyurethane, can be avoided by optimizing the weight percentages of silk hydrogel itself to make its rheological and mechanical properties at par with the native NP tissue. The reinforcement of salt leached silk scaffolds by silk particles could improve the specificity, pH~5.4), in turn differentially regulating the ERK and SMAD signaling pathways downstream [29]. GlcNAc also upregulates TGF-β1 up-regulation could increase the production of GAG in a dose-dependent manner in both 2D and 3D hydrogels seeded with articular chondrocytes [30]. In mesangial cells, GlcNAc mediated TGF-β1 up-regulation could increase the production of specific ECM components, possibly through the hexosamine pathway that exogenously supplied glucose [31–33]. Thus a controlled delivery of glucosamine in an optimum concentration is required for enhanced GAG production that can be beneficial for treating degenerated IVDs. Further, generation of insights about the mechanism through which this GlcNAc-mediated switch like behavior is caused can help to device strategies for degenerated IVD treatment.

The implantation of autologous NP cells for IVD regeneration is not feasible, as the procedure requires puncturing the annulus fibrosus (AF) tissue. This damage may lead to further degeneration of adjacent discs [34]. Thus, human adipose derived stem cells (hADSCs) appear to be the best candidates for this purpose owing to their easy isolation from liposuctioned waste fat tissue. Moreover, the high proliferation rate of hADSCs helps in achieving a significant population of cells for implantation. ADSCs have reportedly been differentiated towards the NP phenotype either in the presence of TGF-β [35–37] or when co-cultured with NP cells [38], and are thus a potent candidate for NP tissue engineering. Recently, we reported that microgels composed of collagen type II and hyaluronan helped in the differentiation of ADSCs towards a NP-like phenotype by providing a native-like NP microenvironment to the cells [39].

Thus, in the present study we introduce a silk fibron hydrogel-based biomaterial delivery platform, consisting of silk fibron hollow microspheres embedded within fibron hydrogel, offering well suited rheological features for injectability, and shape-conformability into defect sites as well as controlled delivery rate. We hypothesized that the hollow silk microspheres, developed using template sacrificial method using polystyrene templates, loaded with GlcNAc can offer a spatiotemporally controlled release of the moieties into the silk hydrogel system, providing the cells with physiologically relevant amounts of GlcNAc for enhanced GAG production.

2. Materials & methods

2.1. Preparation of silk fibron solution

Bombyx mori silk cocoons were provided by Central Silk Technological Research Institute (Central Silk Board), Bangalore, Ministry of Textiles, Government of India. Fibron protein was isolated as mentioned earlier [40,41]. Briefly, 5.0 g of cocoons were cut into small pieces and degummed in boiling water containing 4.4 g Na2CO3 (Sigma) for 30 min. Extracted fibron fibres were given three consecutive rinses in deionized MilliQ water for 15 min each, air dried and then subsequently dissolved in 0.3 M LiBr (Sigma) at 60 °C for 4 h. The silk-LiBr solution was dialyzed against deionized water using Slide-A-Lyzer cassette (Thermo, molecular weight cut off 3500) to obtain a 6% (w/v) solution of fibron protein.

2.2. Fabrication of silk hollow microspheres

Hollow silk microspheres were fabricated using the template based method [42–46]. Commercially available beads (Spherotech, USA) of 0.5 μm, 0.8 μm, 1.4 μm and 4.4 μm were first coated with a 1% w/v poly-L-Lysine solution to impart a positive charge to the polystyrene surface. Following coating, the beads were spun at 4000 rpm for 20 min and then washed with deionized (DI) water by vortexing for 5 min. The beads were then resuspended in 2% w/v silk fibron solution overnight and then washed with DI water twice and incubated with 70% ethanol for 2 h. The beads were then resuspended in DI water and crosslinked with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC)/N-hydroxysuccinimide (NHS) (12 mM EDC, 4.8 mM NHS) in an aqueous solution for 1.5 h and subsequently the reaction was stopped by adding 1 mM Na2HPO4. The beads were washed twice with DI water and the final wash was with 70% ethanol for 30 min. The beads were resuspended in water and the polystyrene core was dissolved by adding tetrahydrofuram (THF) to the solution (1:1 v/v) for 2 min. The silk hollow spheres were then spun down, washed twice with DI water and then with 70% ethanol. The beads were then lyophilized for 24 h at ~80 °C and stored in dried condition at 4 °C for subsequent experiments.

2.3. Sample preparation for scanning electron microscopy (SEM) and transmission electron microscopy (TEM)

The samples were vacuum dried and coated with gold using a gold sputter coater (EMITECH K550X, UK) at 25 mAmp for 1 min, to form a 15–20 nm thick coating. The samples were imaged on a Hitachi S-4700 scanning electron microscope. For TEM (Hitachi H7500 TEM), the samples were taken in DI water suspension and loaded onto the TEM grid for subsequent imaging.

2.4. Analysis of polystyrene removal

To confirm complete removal of polystyrene core from the microspheres following THF treatment, an Attenuated Total Reflectance-Fourier Transform Infrared Spectrometer (ATR-FTIR) analysis of the spheres was performed after crushing them in a mortar and pestle to expose any traces of polystyrene present within the core of silk microspheres. Particular attention was given to the characteristic peaks of polystyrene and silkworm in the obtained spectrum. Uncoated polystyrene beads and hollow silk fibroin spheres were examined after vacuum drying on Varian 660-IR ATR-FTIR instrument.

2.5. Size and zeta potential analysis

The particle size and zeta potential of silk hollow microspheres were analyzed by using dynamic light scattering (Zeta sizer, Nano-ZS90, Malvern). The DLS measurements were performed with a detection angle of 90° at a wavelength of 630 nm at room temperature. For particle size analysis, the silk hollow microspheres were
suspended in DI water and sonicated (15% amplitude for 12 min with a 1 min ON/OFF pulse) to obtain a uniform suspension. The stability and dispersity of silk hollow microspheres were analyzed by zeta potential values and polydispersity index (PDI).

2.6. Stability of silk hollow microspheres

In order to check the stability of the silk hollow microspheres, the particle size and zeta potential was determined. The stability of the samples was checked in both phosphate buffer saline (PBS) and Dulbecco’s Modified Eagle’s Medium (DMEM) up to five days at physiological pH and temperature by using dynamic light scattering at specific time points. The stability of the silk hollow microspheres was also evaluated against a range of pH and ionic strength to determine their usability for injecting into NP tissue of bovine IVDs.

2.7. Drug loading and entrapment

Hollow microspheres, weighing 1 mg (prepared using 0.8 μm polystyrene template) were suspended in 300 μl of PBS containing 200 μg/ml N-acetyl-D-glucosamine and agitated for 12 h at room temperature. Following agitation, the solution was centrifuged at 4000 g and the spheres were subsequently given four consecutive washes with DI water. To estimate the amount of N-acetyl-D-glucosamine, the supernatant was collected and analyzed in a spectrophotometer [47]. This resultant amount was then deducted from the initial amount loaded into the microspheres to get the loading efficiency of the silk hollow microspheres.

2.8. In vitro release studies

To determine the release pattern of N-acetyl-D-glucosamine from silk hollow microspheres, 1 mg of N-acetyl-D-glucosamine loaded spheres (0.8 μm template) were suspended in 5 ml PBS and incubated at 37 °C. The solution was centrifuged every hour and the supernatant was collected. Next, the 0.8 μm template silk hollow spheres were treated with N-acetyl-D-glucosamine. The resultant amount was then deduced from the initial amount loaded into the microspheres to get the loading efficiency of the silk hollow microspheres.

2.9. Rheological characterization

The flow properties of hollow microsphere dispersions in silk hydrogels were characterized using an AR2000 rheometer (TA Instruments). The measurements were performed using flat plate geometry (20 mm diameter) at 25 °C with 500 μm of gap distance. Firstly, oscillatory stress and strain sweep analysis were performed to establish the linear viscoelastic region for every sample. Subsequently, the storage modulus (G′), loss modulus (G″) and tan (δ) were determined by observing an oscillatory time sweep test for 10 min at a constant frequency of 1 Hz and constant stress of 1 Pa. The effect of ionic strength on gel strength was determined by carrying out a stepped stress of 1 Pa. The effect of ionic strength on gel strength was determined by carrying out a stepped release profile of N-acetyl-D-glucosamine.

2.11. Load testing measurements

Static compression tests on unconfined bovine caudal (tail) IVDs were performed using a Zwick-Roell servo-hydraulic testing instrument with a 1000 N load cell. Previous studies have shown that bovine IVDs act as a good biomechanical model system for human IVDs [60]. Bovine IVDs were brought form a local slaughter house on the day of slaughter and stored at 193 K. The adjacent residual tissue was removed followed by cutting the endplate with a precision electronic saw. A method demonstrated by Saunders et al. was used to prepare degenerated IVDs by injecting collagenase to produce a hollow space in the NP tissue [51]. To induce degeneration in the bovine IVDs, 0.15 ml of collagenase type II (10 g/L) was injected into them and incubated for a period of 18 h at 37 °C. The hollow tissue free spaces in the bovine IVDs created by collagenase treatment were then injected with silk hollow microspheres incorporated silk hydrogels. From now on, collagenase treated samples will be referred to as degenerated samples. The bovine IVDs were tested for their cyclic compressive properties in four batches: normal, degenerated, injected with 1% silk hydrogel and injected with 2% silk hydrogel with silk hollow microspheres. The normal IVD samples were taken as such after preparation while the degenerated IVD samples were injected with 1% PBS into their hollow tissue free spaces. A strain of 0.3% was applied on the samples during the load testing experiments. The first cycle of loading and unloading was considered as the conditioning cycle. The loading and unloading cycles were used to record the stress and strain data. Each loading and unloading cycle was of 1 min duration. Every sample was tested for five complete cycles. The first cycle constituted the conditioning step while all the subsequent cycles had a 20 mm gap in between as the relaxation time. The normal (non-degenerated) IVD and the degenerated IVD were taken as controls.

2.12. Culture of hADSCs on silk-in-silk system

Expanded hADSCs at a density of 1.5 × 10^5 cells/gel (0.5 ml) were mixed with the silk-in-silk hydrogel systems consisting of 3% w/v of 1120 ± 24 mm (average diameter) silk hollow microspheres in 2 w/v% silk hydrogel. High glucose DMEM supplemented with 5% PBS and 1% penicillin-streptomycin was used for the culture of cell laden hydrogels. The solution was then incubated at 37 °C with 5% CO₂ for 24 h at a concentration of 5 mg/ml was added on the third day of culture in the test groups. A complete description of the test and control groups used for the experiment is provided in Table 1; which are thereafter mentioned in the figures in the same format.

2.13. Biochemical analysis

Cell laden hydrogels (n = 5) digestion was carried out with proteinase K solution. GAG content was determined by reacting with phenylmethylsulfonyl blue and the products were measured spectrophotometrically. Chondroitin sulphate-A sodium salt from bovine trachea (Sigma, Aldrich, Ireland) was taken as a standard. Hydroxyproline assay was used to measure total collagen content. Cell laden hydrogels were digested with 500 μL of 1 mg/mL proteinase K (including proteinase inhibitor) and incubated overnight at 56°C. The samples were then boiled at 100°C for 10–15 min. A 1:5.1 volume ratio of 6 N HCl was then used to hydrolyze the samples and incubated at 110°C overnight. Hydroxyproline assay was then used to analyze the dried samples with hydroxyproline taken as a standard (n = 5).

2.14. Gene expression analysis

Cell laden hydrogels were disrupted in Trizol (Invitrogen) and total mRNA was extracted as per the manufacturer’s protocol (RNeasy micro kit, Qagen, UK). mRNA was quantified following the manufacturer’s protocol using Qubit® RNA assay kit (Life Technologies). Reverse transcription was done using Improm-II™ RT system (Promega, UK) and MJ Research PTC 200 Thermal Cycler (Thermo Scientific) as per the manufacturer’s protocol. SYBR Green method was adopted for Quantitative real-time polymerase chain reaction (RT-PCR). The expression of collagen type II (Q000045938), aggrecan (Q00001365), SMAD-3 (Q00008729) and MAPK-11 (Q00010104) were quantified using Quantitect primer assay (Qagen) and were measured using a fluorometer (S. Murab et al. / Biomaterials 55 (2015) 64–83).

Table 1

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Experiment</th>
<th>Conditions</th>
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<tbody>
<tr>
<td>1</td>
<td>Test</td>
<td>1 mM N-acetyl-D-glucosamine + 5 mg/ml TGF-b</td>
</tr>
<tr>
<td>2</td>
<td>Test</td>
<td>1 mM N-acetyl-D-glucosamine + No TGF-b</td>
</tr>
<tr>
<td>3</td>
<td>Test</td>
<td>100 μM N-acetyl-D-glucosamine + 5 ng/ml TGF-b</td>
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<tr>
<td>4</td>
<td>Test</td>
<td>100 μM N-acetyl-D-glucosamine + No TGF-b</td>
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<tr>
<td>5</td>
<td>Control</td>
<td>No N-acetyl-D-glucosamine + 5 mg/ml TGF-b</td>
</tr>
<tr>
<td>6</td>
<td>Control</td>
<td>No N-acetyl-D-glucosamine + No TGF-b</td>
</tr>
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were considered as statistically significant. The relative expression levels of the genes were calculated using the 2^(-ΔΔCt) method.

2.15. Histochemistry and immunofluorescence studies

After 21 days of culture, cell laden hydrogels were fixed with 10% formaldehyde for 4 h, washed with PBS and were fixed in OCT medium and cryosectioned into 8 μm thick sections. The sections were then stained with hematoxylin and eosin (H&E) to check cellular morphology. Safranin-O and alcian blue staining were used to visualize the extracellular GAG accumulated on the scaffold. Sections, 5 μm and 8 μm thick, were collected from each cell laden hydrogel and blocked by 10% bovine serum albumin for 30 min at room temperature, and incubated with primary antibodies against collagen type II (COL2/ab185430, abcam), SMAD3 (ab31451/abcam), mitogen activated protein kinase 11 (MAPK11/ab37793, abcam) and aggrecan (ACAN/ab3773, abcam). 4', 6'-diamidino-2-phenylindole (DAPI) was used to stain the nucleus. Alexa Fluor® 488 chicken anti-rabbit IgG (Invitrogen) and Alexa Fluor 555 goat anti-mouse IgG (Invitrogen) secondary antibodies were used for fluorescent labeling. The sections were given three consecutive washes with PBS. Following this, specimens were rinsed twice with PBS/Tween and the sections were subsequently mounted onto slides. Immunostained sections were examined using a Confocal Laser Scanning Microscope (LSM510, Zeiss, Jena, Germany).

2.16. Digital image analysis

For image analysis, 5 randomly selected regions from immunohistochemistry micrographs were analyzed independently for each respective sample, one representative image has been shown. Color de-convolution technique was used to unmix the pure safranin-O/alcian blue stained areas from nuclear and background stains leaving a complementary image. The pixel intensities of separated safranin-O/alcian blue images range from 0 to 255. The darkest shades of the color were represented by value 0 while 255 represented the lightest shade of the color in the image. Automated score was assigned by observing and measuring the pure safranin-O/alcian blue staining pattern, histogram profile of every image using Image J standard program feature. Histogram profile represents the number of pixels of a specific intensity value against their respective intensity. Therefore, depending upon the intensity of the color score in the images, categorization into high positive, positive, and negative zones was determined.

2.17. Statistical analysis

Data have been presented as mean ± SD. The number of replicates has been indicated with the respective methods. Single factor paired one tailed students t-test was used for the analysis of the significance of variations in loading efficiency. Two-way ANOVA followed by Fischer’s post-hoc analysis was used for the analysis of the significance of the variations for all other experiments. The difference between different test and control groups was calculated by p value. The groups with p < 0.05 were considered as statistically significantly different.

3. Results

3.1. Particle size and surface potential

Silk hollow microspheres were fabricated using polystyrene template via template sacrificial method. Templates of sizes 4.4 μm, 1.4 μm, 0.8 μm and 0.4 μm yielded hollow spheres with respectively increasing sizes (Fig. 1A). The polydispersity index showed that the hollow spheres were uniformly dispersed in the media (Table 2). The zeta potential of the hollow microspheres was –53 ± 4.1 mV. Zeta potential of the silk hollow microspheres were found to be slightly lower in PBS, while the values dipped further down in DMEM media (Fig. 1B).

3.2. Removal of polystyrene template

The complete removal of the polystyrene core was confirmed by ATR-FTIR spectroscopy (Fig. 1C). The polystyrene beads showed prominent peaks of 3003 cm⁻¹ and 2926 cm⁻¹ for aromatic CH stretching vibration, 1528 cm⁻¹ and 1453 cm⁻¹ for C–O–C symmetric and anti-symmetric stretching vibrations, 754 cm⁻¹ and 700 cm⁻¹ for aromatic CH deformation vibration. After dissolving polystyrene beads, the silk hollow microspheres were analyzed by ATR-FTIR. The samples did not show any peaks for polystyrene (1528 cm⁻¹, 1453 cm⁻¹, 754 cm⁻¹, 700 cm⁻¹, 754 cm⁻¹ and 700 cm⁻¹) while prominent characteristic peaks for amide I at 1630 cm⁻¹ (signifying C=O stretching), 1520 cm⁻¹ (signifying amide II (secondary NH bending)) and 1250 cm⁻¹ (signifying amide III) were observed. The FTIR data confirmed the complete removal of polystyrene core from the beads and subsequent formation of hollow microspheres which were confirmed by SEM and TEM.

3.3. Morphological analysis of hollow microspheres

The polystyrene beads showed a smooth and round morphology of homogenous shape and size (Fig. 1D). The polystyrene beads were coated with poly-L-Lysine to mask the negative charge of the beads as the surface positive charge which will be conducive for silk fibrin attachment (Fig. 1E). The poly-L-lysine coated beads showed a spiked surface due to the self-assembly of the peptide on the polystyrene beads confirming the success of the coating step. The poly-L-lysine coated beads were then coated with silk fibrin and subsequently the core was dissolved with THF under alkaline conditions to make them hollow (Fig. 1F). The SEM images of hollow silk fibrin microspheres showed a round morphology of homogenous size and shape. SEM analysis also showed some sectioned hollow spheres, where the hollow cavity inside the spheres was visible (Fig. 1G). The hollow silk microspheres were then visualized by TEM that clearly depicted a perfect round morphology and the presence of a hollow cavity inside the spheres (Fig. 1H). The thickness of the silk shell as measured by SEM images using Image J software was found to be 17.46 ± 5.02 nm. The ruptured silk hollow microspheres visualized during morphological analysis through SEM were used for evaluating the shell thickness of these particles.

3.4. Stability of silk hollow microspheres

The silk hollow microspheres were synthesized for their intended use in controlled drug delivery in the IVD which has a significantly high ionic strength (~400 mM) as compared to the other body fluids (~120 mM) [52]. During the process of disc degeneration, the pH of the disc was altered with each consecutive degradation step. These conditions generated the need for testing the stability of the silk hollow microspheres under a range of ionic strength and pH. The increase in average particle size will signify the aggregation of the hollow microspheres which will be due to a corresponding drop in the zeta potential because of the surface charge alterations of the microspheres relating to varying ionic strengths and pH. The hollow microspheres were tested on a range of 100–1000 mM of NaCl. The zeta potential of the hollow microspheres varied from ~21.3 mV to ~50.1 mV (Fig. 2A). While the average size remained more or less stable (data not shown) which indicated that the particles are fairly stable at a wide range of ion concentrations and can withstand the microenvironments prevailing in the IVD. The hollow microspheres were further tested for their stability in a range of pH solutions ranging from 3 to 11 pH. The zeta potential of the hollow microsphere varied from +26.28 mV to ~37.92 mV (Fig. 2B). The potential, though changing from negative to positive, still remained considerably high which prevented the aggregation of the particles, reflected in the average size of the particles which remained unaffected throughout the whole pH range (data not shown).

3.5. GlcNAc loading studies

The loading efficiency was analyzed by quantifying GlcNAc in the supernatant. The maximum GlcNAc loading capacity was determined by keeping the amount of GlcNAc constant while taking different sizes of silk hollow microspheres for loading (Fig. 2C).
Fig. 1. (A) Particle size determined by DLS in water, PBS and complete media for silk hollow spheres synthesized by polystyrene templates of size 4.4, 1.4, 0.8 and 0.5 μm. The size of silk hollow microspheres remained constant in the three solutions indicating their stability and usability under physiological conditions. (B) Zeta potential values of silk hollow spheres prepared on 0.8 μm polystyrene templates in water, PBS and media at day 1–5. This indicated that the hollow microspheres will remain stably dispersed under physiological conditions. (C) FT-IR spectra of silk hollow sphere and polystyrene template control showing complete removal of the template during the dissolution step. Polystyrene removal is necessary to avoid any adverse immune response or cytotoxicity against the hollow spheres. SEM images of the particles during different stages of fabrication: (D) polystyrene template, (E) PLL coated templates, (F) Hollow silk microspheres, (G) Magnified image of hollow microspheres showing a microsphere with broken wall exposing the spherical cavity inside the microspheres. (H) TEM image of hollow silk microspheres showing the hollow core of the microspheres and the perfect rounded morphologies. Values are given as mean ± standard deviation.
This observation suggested that the loading efficiency remained constant after 0.8 μm size, so this particular size was chosen for further experimentation. The 0.8 μm microspheres were then loaded with different amounts of GlcNAc to assess the optimum loading concentration. It was found that the loading efficiency became constant after 1 mg/ml and hence these spheres were chosen for further analysis.

3.6. In vitro release studies

Upto 96 h, no significant differences were observed in the release pattern from any of the respective particle sizes. Total amount of GlcNAc released from the spheres was 8–10 μg. At 216 h, the release profile showed significant differences between different sizes (p < 0.01, n = 3) as the release of GlcNAc from the 0.8 μm and 1.4 μm template hollow microspheres showed higher release than the 0.5 μm hollow microspheres (Fig. 2E). GlcNAc release increased significantly after the exposure of the hollow spheres to enzymatic digestion (Fig. 2F). Thus, spheres made with 8 μm polystyrene bead templates were used for further experimentation owing to their higher release profiles which were most likely a result of their higher surface area available for the release of the molecules. Approximately 80.5% and 64.2% of release was observed for pro tease and collagenase treated hollow microspheres, while the untreated spheres showed only 10.1% release profile at 96 h.

3.7. In vitro cytotoxicity studies

In vitro cytotoxicity of the silk hollow microspheres was tested on monolayer of hADSCs and bovine NP cells. All the four groups showed negligible cytotoxicity with silk hollow microspheres as reflected by alamarBlue® assay (Fig. 3A, H). A similar trend was observed when the cells treated with silk hollow microspheres were tested for cell membrane integrity with LDH release profile (Fig. 3B, I). The Live/Dead assay showed a nominal and almost equivalent number of dead cells in all the four treatment groups after 7 days of treating the cells with silk hollow microspheres (Fig. 3 hADSCs: C,D,E,F,G/NP: J,K,L,M,N).

Cell viability of NP cells (2 × 10⁶ cells/well) with different amounts of silk hollow microspheres at day 7 (Fig. 3H) demonstrated negligible cytotoxicity with silk hollow microspheres. Membrane integrity of the NP cells (measured using LDH release profile) with different amounts of silk hollow microspheres (Fig. 3I) illustrated no negative effect of the hollow microspheres on the membrane integrity of the NP cells as observed by considerably low LDH release profiles.

3.8. Injectableability of silk-in-silk hydrogel system

The viscoelastic properties of silk hollow microsphere dispersions in silk hydrogels were studied via rheology. Rheological characteristics were performed well within the linear viscoelastic range as confirmed by the oscillatory stress and strain sweeps. tan(θ) values below 1 (Fig. 4E,F) and elastic modulus G' higher than the viscous modulus G'' (Fig. 3A–D) under all the conditions confirmed the gel characteristic of the silk hollow microsphere dispersions in silk hydrogel system. A general increase in the elastic moduli of gels (2 w/v %) with increasing ionic strengths was observed (Fig. 4A), which can be explained by effective packing of the silk fibroin chains due to salt induced self-assembly process at high ionic strengths. A similar trend was observed with increasing pH values (Fig. 4B). The increase in pH may have resulted in an increased self-assembly process of silk which subsequently resulted in a corresponding increase of elastic modulus. The viscosity of silk microsphere-in-silk hydrogel system was measured as a function of shear rate to determine the injectability of the system. Shear-thinning behavior was observed for silk-in-silk hydrogel systems as a result of increasing shear rate (data not shown). At high shear rates, the cohesive forces resulting from the tight silk fibroin chain packing and attractive electrostatic interactions were destroyed which allowed for the injection of the silk-in-silk system through the syringe as a result of low viscosity. As shown in Fig. 4H, the gel was transformed into a liquid–like material with considerably low elasticity (G') as a result of the destruction of the gel network system at higher (1000% for 1 min) shear strain. Gel rigidity was restored to 70% of its original G' value: within 30 s of gel destruction (Fig. 4I). Whereas within 5 min, up to ~90% of the viscosity was recovered as compared to that of the original value. Silk-in-silk hydrogel system is thus apt for injection and extrusion through syringes owing to its self-healing and shear-thinning behavior.

3.9. Cyclic compressive testing of degenerated IVDs

A good hysteresis of the stress vs strain was shown by the normal (nondegenerated) IVD (Fig. 5A). This type of stress–strain behavior in bovine IVDs is due to the difference in fluid inflow–outflow rate and strain rate [50]. The final stress increases with each successive cycle during the cyclic compression tests, which is due to the partial dehydration caused by the applied strain that results in an increased tissue volume fraction within the tissue. A significant decrease in the maximum stress value was observed in the case of degenerated IVDs injected with PBS and served as negative control for the study (Fig. 5B). The reswelling ability of the degenerated bovine IVDs during the observed period was significantly reduced as a result of the experimental induced degeneration procedure. Fig. 5C,D shows average stress vs strain data for degenerated bovine IVDs containing 1% and 2% silk hydrogel respectively. Importantly, injection of silk hydrogels in the degenerated IVD caused an increase in maximum stress and a more pronounced hysteresis similar to the native IVDs (Fig. 5C,D) in concentration-dependent manner.

3.10. Cellular morphology studies (SEM)

Human ADSCs (1.5 × 10⁶) were seeded on silk-in-silk hydrogel system consisting of 5% w/w of 1120 nm silk hollow microspheres loaded with 120 μg of GlcNAc/mg in 2% w/v silk hydrogel. The hADSCs showed an extended morphology on the hydrogel surface and were covered with matrix materials in all the samples (Fig. 5 E–J).

3.11. sGAG estimation

The sulphated GAG content analyzed at day 21 showed a marked difference in the GAG produced in different groups (Fig. 5K). All the four experimental groups with GlcNAc produced statistically significant higher amount of GAG as compared to the control groups that were devoid of GlcNAc. Interestingly, the test groups with 1 mM GlcNAc showed statistically significant higher GAG production than the group with 100 μM GlcNAc (p < 0.01). Surprisingly, the groups

<table>
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<th>Sample</th>
<th>Particle size (μm)</th>
<th>Polydispersity index</th>
<th>Zeta potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.4</td>
<td>4.61 ±0.02</td>
<td>0.04 ±0.01</td>
<td>-55.5 ±3.2</td>
</tr>
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<tr>
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without TGF-β1 demonstrated significantly higher GAG production over those with TGF-β1. This suggested that TGF-β1 may be impeding the GAG production from the hADSCs by a positive feedback response.

3.12. Collagen estimation

The collagen content of the hADSC cultured in the silk-in-silk hydrogel system was analyzed at days 11 and 21 respectively.

Fig. 2. Stability of silk microspheres at different ionic concentrations (A) and pH values (B). The zeta potential of the microspheres varied with ionic strength and pH of the solution but remained significantly high enough which is necessary to prevent microsphere aggregation in the high pH and ionic conditions of the degenerated NP tissue. (C) Loading efficiency of GlcNAc in different silk hollow microspheres with varying sizes after 12 h of incubation (*p < 0.001, n = 5). The loading efficiency of the spheres became approximately constant from 0.8 μm templated spheres, so the same were chosen for further experimentation. (D) Loading efficiency of GlcNAc in 0.8 μm silk hollow microspheres with varying loading concentrations after 12 h of incubation (*p < 0.01, n = 5). The release profile of GlcNAc from silk hollow microspheres of different sizes (E). Release profile of 0.8 μm templated silk hollow microspheres in the presence of collagenase and protease enzymes (F). The enzymes increased the rate of release of GlcNAc from the microspheres as a function of the sphere degradation rate.
Fig. 3. Cell viability of hADSCs (20,000 cell/well) with different amounts of silk hollow microspheres at day 7 (A). The silk hollow microspheres posed negligible cytotoxicity. Membrane integrity of the hADSCs cultured as LDH release profile at 20,000 cells/well seeding density with different amounts of silk hollow microspheres (B). There was no negative effect of the hollow microspheres on the membrane integrity of the hADSCs as depicted by very low LDH release profiles. Live & Dead cell assay with 25 μg (C), 50 μg (D), 100 μg (E), 200 μg (F), and PBS (G) of silk hollow microspheres of hADSCs at 10,000 cell/well seeding density. (Green: live cells, red: dead cells/Scale bar 200 um). The values are given as mean ± standard deviation. Cell viability of NP cells (20,000 cell/well) with different amounts of silk hollow microspheres at day 7 (H). The silk hollow microspheres posed negligible cytotoxicity. Membrane integrity of the NP cells cultured as LDH release profile at 20,000 cells/well seeding density with different amounts of silk hollow microspheres (I). There was no negative effect of the hollow microspheres on the membrane integrity of the NP cells as depicted by very low LDH release profiles. Live & Dead cell assay with 25 μg (J), 50 μg (K), 100 μg (L), 200 μg (M), and PBS (N) of silk hollow microspheres of NP cells at 10,000 cell/well seeding density. (Green: live cells, red: dead cells/Scale bar 100 um). The values are given as mean ± standard deviation. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
Fig. 4. Elastic modulus $G'$ (A), Storage modulus $G''$ (C) and tan (δ) (E) profiles of silk-in-silk system with varying ionic concentrations. Elastic modulus $G'$ (B) and Storage modulus $G''$ (D) and tan (δ) (F) profiles of silk-in-silk system with varying pH. The $G'$ remained greater than the $G''$ under all conditions showing the systems injectability and hydrogel property under these conditions. This is required for the use of this system under the low pH and high ionic conditions prevailing in the degenerated disc. Gel recovery after gel destruction assessed by monitoring $G'$ and $G''$ as a function of time: initial gel strength (G), gel destruction (H), and gel recovery (I), respectively. The silk-in-silk hydrogel system demonstrated a network destruction occurring during the injection step which automatically recovered (approximately 70%) and formed a hydrogel within 30 s of gel destruction. The gel recovered to a 90% of its original state within 5 min regaining its normal gel structure and rheological properties necessary for dissipating the mechanical forces put on the disc.
A similar trend was followed for sulphated GAGs as observed in collagen content. All the four experimental groups with GlcNAc produced higher amount of collagen as compared to the control groups that were devoid of GlcNAc. This clearly suggests that GlcNAc helps in enhanced production of GAG molecules. Secondly, the test groups with 1 mM GlcNAc showed significantly higher collagen production than the group with 100 μM GlcNAc (p < 0.01). This result suggested that a controlled release of 1 mM GlcNAc had a more pronounced effect on collagen production as compared to 100 μM GlcNAc. It was also observed that the groups without TGF-β1 were having higher collagen deposition as compared to those with TGF-β1 again suggesting the putative effect of TGF-β1 on collagen production from the hADSCs through a positive feedback control.

3.13. Gene expression analysis

Gene expression analysis (Fig. 6 A–D) carried out by qRT-PCR showed significantly enhanced expression of COL II, ACAN, SMAD-3 and MAPK-11. The control groups in general showed significantly low expression of SMAD-3, MAPK-11, COL II and ACAN. All the markers namely COL II, ACAN, SMAD-3 and MAPK-11 showed significant higher expression in samples with 1 mM GlcNAc (COL II-4.6 fold upregulated, ACAN- 3.5 fold upregulated, SMAD-3-4.3 fold upregulated and MAPK-1- 3.6 fold upregulated) as compared to those with 100 μM GlcNAc (COL II-3.5 fold upregulated, ACAN- 2.5 fold upregulated, SMAD-3-3.6 fold upregulated and MAPK-1- 3 fold upregulated). This was in agreement to all other biochemical and histochemical analysis mentioned earlier.

3.14. Histology

3.14.1. H&E staining

On visual examination, H&E staining showed drastic increase in the number of cells in all the samples but variations were visible among matrix deposition (Fig. 6 E–J). All the test groups showed higher matrix accumulation as compared to the control groups without GlcNAc. The tests groups also showed variations in matrix deposition consistent with the findings of the biochemical assays for sGAG and collagen.

3.14.2. Safranin-O and alcian blue staining

Safranin-O (Fig. 7 A–F) and alcian blue (Fig. 8 A–F) staining were performed to visualize the sulphated GAGs deposited by the hADSCs in the silk-in-silk hydrogel systems. Both the staining results indicated GAG accumulation in the silk-in-silk hydrogel system. The test groups of 1 mM and 100 μM GlcNAc showed higher accumulation of GAG molecules as compared to the controls that were devoid of GlcNAc suggesting that GlcNAc enhances the production of GAG molecules in hADSCs. While the test groups with TGF-β1 showed lower GAG deposition as compared to those without TGF-β1.

3.15. Immunohistochemistry

Immunohistochemical staining confirmed the presence of collagen type II and aggrecan (Fig. 9 A–F & G–L) in all the groups and the intensities followed a similar trend observed in biochemical and histochemical estimations, as quantified by Image J for fluorescence intensity (Fig. 9 M, N). MAPK-11 and SMAD-3 specific staining showed relatively higher intensities in the test groups as compared to the control groups without GlcNAc (Fig. 10 A–F & G–L) and the fluorescence intensity (quantified using Image J) demonstrated (Fig. 10 M, N) a trend similar to that for biochemical estimation for GAG. This clearly indicated that GlcNAc trigger these two pathways, thus upregulating their expressions in the test samples where GlcNAc treatment was given.

4. Discussion

Silk based biomaterials have been earlier used in various forms for sustained drug delivery purposes [53]. The tunable release rates of drugs from silk biomaterials via diffusion and biodegradation makes them a suitable candidate for drug delivery applications. GlcNAc has been administered orally for treating osteoarthritis at large [54] but its mechanism of action for production of extracellular matrix to improve degenerated cartilaginous tissue is not clearly understood. The outcomes of the clinical studies carried out to understand the effect of GlcNAc remains controversial due to the small sample size and relatively small follow-up durations [54]. The present study demonstrated that the controlled delivery of 100 μM and 1 mM of GlcNAc from injectable hollow silk fibroin microspheres embedded in silk hydrogel enhanced GAG production in the hADSCs as compared to the controls without GlcNAc. Silk microsphere-in-silk hydrogel system proposed in this study is a purely biomaterial based system that holds potential to release bioactive molecules in a spatiotemporally controlled fashion to increase GAG production in the degenerated disc and may eventually restore the biomechanical properties of the degenerated disc.

A template sacrificial method was used to synthesize silk hollow microspheres [42–46]. Polystyrene microbead templates used for the fabrication of the hollow spheres are negatively charged, just like silk at pH 7 (pI 3.9). Therefore, a surface charge modification strategy was utilized to make the beads conducive for incorporating fibroin polymer chains by coating them with poly-L-lysine. The polystyrene templates were finally dissolved by THF treatment as it exhibits strongly polarisable side groups, which strongly interacts with solvents containing aromates or strongly polar groups. When covered with silk, THF could easily diffuse through the silk polymer network and dissolve the polystyrene template. But this treatment inflicted detrimental effect on the silk protein due to which the spheres were found to collapse. The problem was solved by treating the spheres with 70% ethanol to β-crystallize the silk fibroin chains to induce stability followed by subsequent dissolution of the template under alkaline conditions. Ethanol induces dehydration in fibroin protein chains leading to enhancement of hydrophobic interactions prevailing inside the secondary and thus driving it towards the β-sheet crystallized structure imparting stability. Whereas the role of alkaline environment in dissolution step is that it induces the breakage of the extra silk chains present in the solution which tend to aggregate the hollow spheres. Thus these two modifications resulted in formation of a clear suspension of silk hollow microspheres. The resultant zeta potential –53 ± 4.1 mV of the silk hollow microspheres was significantly high to make the microspheres stable and prevent aggregation, as reported earlier for silk particles [55]. Whereas, the values were considerably lower in PBS and DMEM media which may be attributed to the salting effect exerted by the buffer solution in the case of PBS and the shielding effect of the serum proteins in media.

The IVD has a higher ionic strength of around 400 mM (as compared to 120 mM of other body fluids) that helps it to maintain high hydrodynamic pressure due to osmotic gradient [48]. High ionic strength may destabilize microspheres and induce subsequent aggregation due to reduction in zeta potential as a result of the salting effect. The intrinsically high zeta potential of the hollow silk microspheres masked the effect of very high ionic concentrations (up to 1 M) thus preventing their aggregation. Another critical
Fig. 5. Panels showing average stress vs strain data for IVDs showing restoration of mechanical properties of the degenerated discs after injection with the silk-in-silk hydrogel system. Average data for IVDs containing PBS and also normal IVDs are shown for comparison. (A) native IVD, (B) degenerated IVD (with PBS control), (C) with 1% silk hydrogel, (D) with 2% silk hydrogel. The native IVDs behaved in a perfect viscoelastic pattern showing a constant hysteresis curve. The degenerated control discs injected with PBS showed a 2 fold reduction in stress-strain hysteresis and ultimate compressive strength. The silk-in-silk hydrogel system restored the compressive properties of the degenerated disc. 1% silk hydrogel system demonstrated an increase in the compressive strength but the hysteresis curve was not complete showing deviated viscoelastic behavior. While the 2% silk-in-silk hydrogel system demonstrated an increase in the compressive strength but the hysteresis curve was not complete showing deviated viscoelastic behavior.
Fig. 6. Quantitative Real Time-PCR studies to evaluate gene expression in hADSCs cultured within silk-in-silk hydrogel system: collagen (A), aggrecan (B), MAPK11 (C), SMAD3 (D) (statistical significance between groups: *p < 0.001,**p < 0.01, n = 3). Histochemistry of the cell laden hydrogels after 21 days of hADSCs culture: H&E staining and 3D surface plot of IHC stained images showing deposition and distribution on the cell laden hydrogels-E (Gln 1 mM + TGF), F (Gln 1 mM + No TGF), G (Gln 100 μM + TGF), H (Gln 100 μM + No TGF), I (TGF + No Gln), J (No Gln + No TGF). Scale bar for E,F,G,H,I,J 100 μm.

hydrogel system recovered the compressive strength of the degenerated discs at par with the native discs. SEM images of cell laden hydrogels after 21 days of culture- E (Gln 1 mM + TGF), F (Gln 1 mM + No TGF), G (Gln 100 μM + TGF), H (Gln 100 μM + No TGF), I (TGF + No Gln), J (No Gln + No TGF). Scale bar for A–F 10 μm. All the groups showed good proliferation of hADSCs on the hydrogels and maintained the native spread morphology of hADSCs. GAG (K) and Collagen (L) estimation of the cell laden hydrogels after 11–21 days of hADSC culture. *statistical significance between groups: *p < 0.001, n = 3). The exogenous addition of TGF-β in conjunction with GlcNAc significantly reduced GAG and COL production as compared to the only GlcNAc provided groups.
physicochemical condition prevailing in the degenerated IVD is the change in pH with each consecutive step of degradation [56]. As the pI of silk fibroin is 3.9, the silk hollow microspheres changed their zeta potentials from negative to positive at pH 3, which was still high enough to avoid aggregation as observed by corresponding particle size readings.

In this study, GlcNAc was loaded onto the silk hollow microspheres using solvent diffusion. The high drug loading efficiency was done by using Image J (NIH) software by thresholding of stained zones of IHC images followed by pixel vs intensity determination by color de-convolution plugin. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 7. Histochemistry of the cell laden hydrogels after 21 days of hADSCs culture: Safranin-O staining, 3D surface plot of stained images showing deposition and distribution of GAG, color de-convoluted image, reference histogram profile and pixel analysis data table scoring. A (Gln 1 mM + TGF), B (Gln 1 mM + No TGF), C (Gln 100 μM + TGF), D (Gln 100 μM + No TGF), E (TGF + No Gln), F (No Gln + No TGF). Scale bar for A–F 100 μm. 3D image reconstruction and software-based analysis data set of constructs representing score was done by using Image J (NIH) software by thresholding of stained zones of IHC images followed by pixel vs intensity determination by color de-convolution plugin. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
obtained can be attributed to the hydrophobicity of the silk fibroin protein which prevents the leaching out of the drug molecules during the process of loading and their controlled release from the hollow microspheres by repelling them and controlling their diffusion out of the microspheres. Effect of hydrophobic nature of silk in higher drug loading efficiency of methotrexate molecules into silk-albumin particles has been reported earlier [57]. The release of GlcNAc was not significant in vitro, so enzymatic

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**Fig. 8.** Histochemistry of the cell laden hydrogels after 21 days of hADSCs culture: Alcian blue staining, 3D surface plot of stained images showing deposition and distribution of GAG, color de-convoluted image, reference histogram profile and pixel analysis data table scoring. – A (Gln 1 mM + TGF), B (Gln 1 mM + No TGF), C (Gln 100 μM + TGF), D (Gln 100 μM + No TGF), E (TGF + No Gln), F (No Gln + No TGF). Scale bar for A–F 100 μm. 3D image reconstruction and software-based analysis data set of constructs representing score was done by using Image J (NIH) software by thresholding of stained zones of IHC images followed by pixel vs intensity determination by color de-convolution plugin. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
Fig. 9. Immunohistochemical analysis of cell laden hydrogels after 21 days of hADSCs culture for presence of: collagen type II: A (Gln 1 mM + TGF), B (Gln 1 mM + No TGF), C (Gln 100 μM + TGF), D (Gln 100 μM + No TGF), E (TGF + No Gln), F (No Gln + No TGF). Scale bar for A-F 100 μm. Sections were stained with an anti-Col II antibody (red) and counterstained with DAPI (blue) for cell nuclei. Aggrecan: G (Gln 1 mM + TGF), H (Gln 1 mM + No TGF), I (Gln 100 μM + TGF), J (Gln 100 μM + No TGF), K (TGF + No Gln), L (No Gln + No TGF). Scale bar for G-L 100 μm. Sections were stained with an anti-Col I antibody (red) and counterstained with DAPI (blue) for cell nuclei. Quantification of cell fluorescence using ImageJ, where IntDen and CTCF represent integrated density and corrected total cell fluorescence, respectively for COL II and Aggrecan (M, N) (statistical significance between groups: *p < 0.001/**p < 0.01, n = 3). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
Fig. 10. Immunohistochemical analysis of cell laden hydrogels after 21 days of hADSCs culture for presence of:- MAPK: A (Gln 1 mM + TGF), B (Gln 1 mM + No TGF), C (Gln 100 μM + TGF), D (Gln 100 μM + No TGF), E (TGF + No Gln), F (No Gln + No TGF). Scale bar for A,B,C,D,E,F 100 μm. Sections were stained with an anti-MAPK antibody (green) and counterstained with DAPI (blue) for cell nuclei while the cytoskeleton (actin) was stained red by rhodamin phalloidin. SMAD: G (Gln 1 mM + TGF), H (Gln 1 mM + No TGF), I (Gln 100 μM + TGF), J (Gln 100 μM + No TGF), K (TGF + No Gln), L (No Gln + No TGF). Scale bar for G,H,I,J,K,L 100 μm. Sections were stained with an SMAD antibody (red) and counterstained with DAPI (blue) for cell nuclei while the cytoskeleton (actin) was stained green with FITC phalloidin. Quantification of cell fluorescence using ImageJ, where IntDen and CTCF represent integrated density and corrected total cell fluorescence, respectively for MAPK and SMAD (M, N) (statistical significance between groups: *p < 0.001/**p < 0.01, n = 3). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
treatment was given to degrade the hollow spheres which resulted in a spatiotemporally controlled release of GlcNAc, simulating the physiological conditions prevailing in the degenerated IVD. This trend was in accordance with our previous study which demonstrated the enhanced controlled release of plasmid DNA from hollow elastin-like polypeptide microspheres [43].

The rheological characterization of the silk-in-silk system demonstrated that the system is injectable and maintains a hydrogel state under a range of ionic concentrations and pH resembling in vivo-like conditions. The hydrogel system showed a general shear thinning behavior. The injectability studies showed that the hydrogel network broke extensively during the injection phase when very high shear rate was applied on the hydrogels, but soon after withdrawing the shear force, the silk hydrogel self-assembled, resulting in restoration of its original rheological properties within 30 s of network destruction. The silk fibroin/polyurethane composite hydrogels prepared by Hu et al. [25] showed a similar rheological profile with G’ values higher than the G’ demonstrating elasticity of the hydrogels. But the composite was hyperelastic when compared to the native NP tissue which can result in biomechanical complications after implantation in vivo, while the present silk-in-silk hydrogel system demonstrated rheological properties similar to native NP tissue [58].

Further, hADSCs culture revealed the effect of GlcNAc on sulphated GAG production in the cell laden hydrogels as a function of its spatiotemporally controlled release from the silk hollow microspheres. Two concentrations of GlcNAc were selected based on prior studies [59], which showed the effect of GlcNAc on chondrocytes and hMSCs. GlcNAc is reported to increase TGF-β production by these cells as well as the number of TGF-β receptors on the cellular surface. TGF-β is also reported to induce pathways for GAG production [30,60]. TGF-β has been found to signal through transmembrane serine/threonine protein kinase receptors [61]. Activation of ligand-receptor then induces the phosphorylation of downstream targets including Smad transcription factors [62,63]. The Smad2 and Smad3 which are activated through receptors (R-Smads), are directly phosphorylated by the activated TGF-β receptor, after which the TGF-β receptor I (TβRI), complexes with co-Smad-Smad4 and is then translocated to the nucleus in order to activate transcription [64]. Pathways like JNK, Erk, p38 and pI3 kinase, NF-κB and Rho GTPase signalling in a Smad independent manner are also activated by TGF-β [65,66]. Susarla et al. demonstrated that GAG production is regulated by the SMAD pathway by targeting selective Smads [67]. The signalling of SMAD 3 and MAPK13 as a result of GlcNAc to trigger GAG production was evident from their upregulated expression in RT-PCR. This confirmed that the controlled release of GlcNAc from the silk hollow spheres provided a physiologically relevant concentration for the induction of chondrogenic differentiation and enhanced GAG production. Notably, groups with exogenously added TGF-β showed decreased GAG production. The immunostaining results also confirmed decreased Smad and MAPK signalling in these groups. This can be explained based on a negative feedback that may exist in TGF-β signalling pathway. Basically the exogenous TGF-β supplied to cells was in addition to the naturally produced TGF-β by cellular metabolism, which due to the copious amounts present may have triggered a negative feedback via Smad 7; known to antagonize TGF-β signalling [68,69], most likely responsible for the lower GAG production in these groups. A schematic illustrating the probable signalling involved in GlcNAc regulation of GAG production is depicted in Fig. 11. Some studies have also shown the involvement of MAPK and NF-κB pathways in regulating the expression of Aggrecanase-1, thus suggesting that the present system can also potentially help in regulating the catabolic activities in the degenerated disc [70] which can be explored in future studies.

Fig. 11. Schematic explaining the cellular signaling and biochemical steps occurring in the cells as a result of GlcNAc addition. Glucosamine provided exogenously gets into the cells and is metabolized to UDP-GlcNAc. The substrate then regulates the pathway resulting in upregulation in TGF-β synthesis. This TGF-β then triggers the GAG synthesis through MAPK and SMAD signaling pathways resulting in enhanced GAG production.
To the best of our knowledge, this is the first study demonstrating the effect of controlled release of physiologically relevant concentrations of GlcNAc on hADSCs. This suggests the potential use of autologous hADSCs in the treatment of degenerated disc diseases in conjunction with GlcNAc controlled release therapy. A previous finding showed a similar trend in ECM production at physiological levels of GlcNAc during short term in vitro studies, which demonstrated that GlcNAc concentration higher than 10 mM can be toxic for chondrocytes [59]. Another study showed the negative effect of GlcNAc on the catabolic effects occurring in degenerative disc diseases like NO production, induction of nuclear factor k beta (NFκB) by IL-1B and expression of cyclooxygenase 2 (COX-2) [60]. GlcNAc administered in high doses has also been shown to be detrimental for metabolic activities in bovine chondrocytes [30], therefore a controlled delivery system developed in the present work can help exploit its positive effects on sulphated GAG production while avoiding the deleterious effects caused by excessive concentrations of GlcNAc on the cells.

The mechanical characterization studies were done on a degenerated model of IVD created by using collagenase digestion of the bovine discs. The load bearing function of IVD is basically regulated by its collagen and aggrecan matrix. Collagen makes the framework of the tissue while aggrecan is the required mechanical stiffness to the tissue. While the aggrecan molecules impart it with its osmotic turgor due to the charged hydrophilic GAGs attached to it that help regulate the exchange of water that helps the tissue to take up physiological loading. Degeneration of the IVD involves the loss of both the collagen framework and GAG molecules thus the swelling and load bearing properties of the tissue are lost. The present silk-in silk system was hypothesized to help bear the mechanical loading of the degenerated IVD through the silk hydrogel. The silk hydrogel mimics the collagen network and thus can help the degenerated tissue in bearing the physiological loadings. The hollow silk microspheres will release GlcNAc in a controlled fashion to increase the GAG production which is a long term treatment strategy. Thus to test the load bearing capacity of the silk hydrogel system, we needed a test model with degenerated collagen matrix. Thus the collagenase degenerated ex vivo IVD system was taken up for cyclic compressive testing as the silk hydrogel can be tested for its ability to replace the collagen matrix to provide the desired mechanical properties to the tissue. There are studies that have taken up models that are based on testing the load bearing capacity of a hydrogel after its osmotic swelling in a high osmolar solution thus mimicking the hydration properties of the tissue imparted by aggrecans [71]. Though this model is capable of mimicking the osmotic situation prevailing in the IVD but is not able to replicate the anatomical environment and loading conditions existing in the degenerated disks. Another study described an injectable hydrogel system based on enzymatically-crosslinked polyethylene glycol and hyaluronic acid embedded with pentosan polysulphate used an in vitro system of agarose gel with a hollow cavity inside [72]. The model was tested for injecting the developed hydrogel but was not taken up for mechanical studies.

The ability of bovine IVDs to reswell up to the native height after the strain removal was significantly reduced as a result of the experimentally induced degeneration. The silk-in-silk hydrogel system injection into the degenerated IVDs helped restore the compressive properties. The 2% w/v hydrogel system was able to restore the compressive profile close to the non-degenerated IVDs as compared to the 1% hydrogel system. Importantly, formation of silk hydrogel after injecting the degenerated IVD caused a decrease in hysteresis and an increase in maximum stress restoring the original mechanical properties. The previously reported silk-polyurethane hydrogels [25] showed a non-linear stress-strain curve like the present hydrogels but the compressive strength of the hydrogels was relatively low which was not at par with the native NP tissue. Secondly, that study lacked a cyclic compressive analysis which only can demonstrate that if the hydrogel is able to restore its height after compression and will help the disc perform its normal function.

We have not considered all the physiological conditions prevailing in the degenerated IVDs during in vitro culture of hADSCs and the mechanical studies did not take into consideration the long-term confined cyclic loading, as both were beyond the scope of this study. However the present study is the first step in devising a silk-in-silk injectable system for the controlled delivery of GlcNAc into the degenerated IVDs in a minimally invasive manner. Thus the silk-in-silk hydrogel system is a potential candidate as a drug and cell delivery platform that can help to retain the native mechanical properties of the degenerated disc by providing proper support; while at the same time help the tissue to regenerate by restoring ECM composition. This system can be modified further by incorporating different cocktails of drug molecules and growth factors in the hollow microspheres. The encapsulation efficiency of the preferred molecule can be increased by fine tuning the charge and porosity of the silk hollow microspheres by different crosslinker concentrations and silk surface modifications.

5. Conclusion

The present study suggests that a spatiotemporally-controlled release of GlcNAc helps in enhancing the chondrogenic phenotype of hADSCs by enhancing the expression of collagen II and aggrecan. GlcNAc promotes the differentiation of hADSCs towards a chondrogenic phenotype by enhancing sulfated proteoglycan accumulation. The silk-in-silk system may offer a potential starting point in restoring the biomechanical functionality of the degenerated discs. The role of MAPK and SMAD signalling in the mechanism of GlcNAc mediated enhancement of ECM production can manifest in the development of a potential therapy for the treatment of degenerative disc disorders.

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